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The Effect of Hypoxia on G Protein Coupled (Opioid) Receptor Gene Expression in Cortical B50 Neurons in Culture

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Abstract: Hypoxia adversely affects cells and tissues, and neuronal cells in particular have been shown to be more susceptible to the injurious effects of hypoxia in which they may begin to die when oxygen supply is reduced or completely eliminated. Opioid receptor agonists have been shown to elicit several central nervous system effects, mediated via G protein-coupled receptors. The aim of this study was to study the effect of hypoxia on G protein coupled receptor gene expression using mu opioid receptor as a case study in cortical neuronal B50 cell lines in culture. The B50 cells were cultured in normoxia (21% O_2; 5% CO_2) and hypoxia (5% O_2; 5% CO_2), and were treated with opioid agonists to determine their effects on hypoxia-induced changes. Three opioid agonists {DAMGO(μ), DSLET(δ) and ICI--199,441(κ)}, were administered to the cells as treatment for 48 hours after 48 hours of initial culture for a total of 96 hours of culture in hypoxic conditions at concentrations of 10, 50 and 100 μM. The levels of G-protein coupled receptor (mu opioid) mRNAs were assessed using RT-PCR. The results showed that hypoxia induced morphological changes in B50 cells in hypoxia while the mu opioid RT-PCR mRNA levels showed no appreciable changes in normal, hypoxic and treated cells. The results show that B50 neuronal cells are susceptible to damage and injurious effects of hypoxia, as are most brain cells and the opioid agonist treatments showed there were no changes in the level of mu opioid receptor gene expression due to hypoxia or agonist treatment in neuronal B50 cells in culture.

Key words: Hypoxia, G-protein coupled receptor, Opioid (mu) receptor, messenger RNA, reverse transcription-PCR, opioid agonist

INTRODUCTION

Cerebral hypoxia is a condition in which there is a decreased oxygen supply to the brain even though there is adequate blood flow (Guyton and Hall, 2005). Neural and brain tissues have been shown to be extremely sensitive to oxygen deprivation and can begin to die within five minutes after oxygen supply has been cut off (Zhang *et al*., 2006; Tomaselli *et al*., 2005). When hypoxia lasts for longer periods of time, it can cause coma, seizures, and even brain death. In brain death, there is no measurable activity in the brain, although cardiovascular functions are preserved (Dietz *et al*., 2003). Hypoxia in the brain may lead to cell death by apoptosis and necrosis. Tomaselli *et al*. (2005) have shown that adenosine, the final metabolic product of in the stepwise dephosphorylation of ATP, is produced and released in the central nervous system in response to ischaemia and hypoxia. Most mammals possess little natural tolerance to severe hypoxia, and their excitable cells and tissues are normally debilitated by any prolonged exposure to hypoxic condition. The primary causes of hypoxia-induced death in mammals are brain dysfunction and cardiac arrhythmias due to a loss of ionic integrity of the cell membranes (Boutilier, 2001). Ion leakage across cell membranes occurs as a result of both intracellular and extracellular ions drifting towards their thermodynamic equilibrium (Rolfe and Brand, 1996; Priebe *et al*., 1996). Cell death occurs when ATP production fails to meet the energetic maintenance demands of ionic and osmotic equilibrium. The rise in free cytosolic intracellular Ca^{2+} concentration results in the activation of Ca^{2+}-dependent phospholipases and proteases that further hasten the rate of membrane depolarisation, leading to uncontrolled cellular swelling and, ultimately, to cell necrosis (Hochachka, 1986; Boutilier, 2001).

Mahura (2003) has shown that neuronal responses to hypoxia can be acute or chronic. In the early stages, neuronal responses to ischaemia-hypoxia are dependent on the modulation of ion channels. Acute responses rely

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mainly on Oxygen-regulated ion channels which mediate adaptive changes in neuron excitability. Energy failure, an early consequence of hypoxia-ischaemia, causes disruption of ionic homeostasis and accumulation of extracellular neurotransmitters. NMDA and AMPA/ kainate receptors and Ca^{2+} channels contribute to excitotoxic neuronal degeneration. Excitotoxicity leads to increased Ca^{2+} influx, which can activate cytotoxic intracellular pathways (Mahura, 2003).

Opioid receptors are found in the central nervous system and are classified as mu (μ), kappa (κ), delta (δ) and sigma (σ) opioid receptors. Opioid receptors are not uniformly distributed in the CNS and are found at areas concerned with pain receptors, with the highest concentration in the cerebral cortex, followed by the amygdala, septum, thalamus, hypothalamus, midbrain and spinal cord (Raynor *et al*., 1996; Chaturvedi *et al*., 2000). The mu receptor has been shown to be high in areas of pain perception and in the medulla, especially in the area for respiration (Reisine and Bell, 1993; Reisine and Brownstein, 1994; Massotte and Kieffer, 1998; Hasbi *et al*., 2000).

The opioid receptors (mu, delta, and kappa) belong to the large family of GPCRs and have diverse and important physiological roles (Piestrzeniewicz *et al*., 2006; Rhim and Miller, 1994). Laugwitz *et al*. (1993) have shown that activated delta opioid receptors are coupled to G_{i1} while activated mu opioid receptors are coupled to G_{i3} in neuroblastoma cells. Mu opioid receptors have been shown to be activated by mu receptor agonists and are coupled through the $G\alpha_{i1}$ and $G\alpha_{oA}$ in human embryonic kidney cells (Saidak *et al*., 2006). Tso and Wong (2000), have shown that both mu and kappa opioid receptors are coupled via both G_i and G_z in HEK 239 cells. The opioid receptors are important targets for thousands of pharmacological agents (Hasbi *et al*., 2000; Wang *et al*., 2007). The stimulation of these receptors triggers analgesic effects and affects the function of the nervous system, gastrointestinal tract and other body systems (Piestrzeniewicz *et al*., 2006). The discovery of opioid peptides including delta-selective enkephalins, kappa-selective dynorphins, and mu-selective endomorphins, which are endogenous ligands of opioid receptors, initiated their structure-activity relationship studies (Fichna *et al*., 2006). The aim of the present work was to investigate the expression of opioid (mu) receptor gene in B50 neuronal cells, to investigate the effect of hypoxia on the expression of mu opioid receptor gene on B50 neuronal cells and to study the effect of mu opioid receptor agonist treatment on opioid (mu) receptor gene in cultured B50 cells using semi quantitative RT-PCR.

MATERIALS AND METHODS

Neuronal culture: One group of B50 neuronal cells were cultured and maintained in a normoxic incubator ($21\%O_2$; $5\%\ CO_2$) as control group and another batch cultured under hypoxia ($5\%O_2$; $5\%\ CO_2$) as experimental group. Cells were cultured in 12-well culture plates for 48 hours and three opioid receptor agonists were treated with opioid agonists to determine their effects on hypoxia-induced changes. Three opioid agonists {DAMGO(μ), DSLET(δ) and ICI--199,441(κ)}, and antagonists were administered to the cells as treatment against hypoxia for 48 hours for a total of 96 hours at a concentration of 10, 50 and 100 μM. The total cellular RNA was extracted from the cultured B50 neuronal cells using the TRIzol reagent method (Invitrogen No 15596-026), as outlined below. The cells were cultured at the cell culture Laboratory of Queen Margaret University Edinburgh and the RT-PCR was done at the Centre for Neuroscience, University of Edinburgh United Kingdom in 2006.

Methods: The B50 cells in different experimental groups were grown and lysed in culture plates by adding 0.5 mL of TRIzol reagent to each well. The cells were homogenized and incubated for 5 min at room temperature. The homogenates were transferred to micro-centrifuge tubes, 0.1 mL of chloroform added, the cap secured and the tubes shaken vigorously by hand for 15 sec. The cellular mixture was incubated at room temperature for 3 min. The mixture was then centrifuged at 12,000 × g for 15 min at room temperature. Following centrifugation, the mixture, separated into 3-layers namely a lower phenol-chloroform phase (Red), a middle interphase (Cloudy) and an upper aqueous colourless phase.

The RNA was present at the upper aqueous colourless phase and formed about 60% of the total volume of the mixture and was transferred to a fresh micro-centrifuge tube. The aqueous phase was mixed with 0.25 mL of isopropyl alcohol and incubated for 10 min at room temperature. The mixture was then centrifuged at 12,000 × g for 10 min at room temperature. At this point the RNA precipitated and formed a gel-like pellet.

The supernatant was removed and the remaining RNA pellet was washed once with 0.5 mL of 75% ethanol and mixed by vortexing. The mixture was centrifuged at 7,500 × g for 5 min at room temperature and the ethanol was decanted. The RNA was then air dried for 10 min, dissolved in 100% deionized formamide and stored at -70ºC to be used in RT-PCR analysis.

Semi-quantitative one step RT-PCR analysis: The semi-quantitative one step reverse transcriptase polymerase chain reaction (RT-PCR) was used to study the expression of mu opioid receptors. Mu Opioid Receptor (MOR) was selected for the semi-quantitative RT-PCR analysis because the MOR was selected for study because opioid receptor subtypes have been shown

to have 68% sequence homology between them and the differences between them was proposed largely, on the basis of radioligand binding studies and as such there is little or no evidence for the presence of the different genes encoding the opioid receptor subtypes (Corbett *et al*., 2006). In some cases receptor heterodimerisation of opioid receptors has been proposed as a possible explanation for the different opioid receptor subtypes (Corbett *et al*., 2006; Milligan, 2004).

The extracted total RNA, Superscript III RT/Platinum Taq Mix, Reaction Mix, 5 nM Magnesium Sulphate, GeneAMP PCR System thermal cycler were used according to the manufacturer's instruction. Mu Opioid Receptor (MOR) sense primers, 5-GGA ACA TGG CCC TTC GGA ACC ATC-3' (574-597) and antisense 5'-TAC CAG GTT GGG TGG GAG AAC GTG-3' (863-840), were selected from Silbert *et al*. (2003), where they were used to study the effect of MOR expression and opioid treatment in myelinated and unmyelinated neurons. Alpha actin primer Sense 5'-GAT CAC CAT CGG GAA TGA ACG C-3' (389bp) and Antisense 5'-CTT AGA AGC ATT TGC GGT GGA C-3', selected from Park *et al*. (1997), where they were used as an internal control for cytoskeletonal study in pericytes.

Programming of the thermal cycler was done as follows: cDNA synthesis 1 cycle at 55ºC for 30 min, Denaturation 1 cycle at 94ºC for 2 min, PCR amplification 40 cycles at 94ºC for 15 sec (Denature), 60ºC for 30 sec (Anneal), 68ºC for 60 sec (Extend), Final extension, 1 cycle at 68ºC for 5 min.

The master mix was prepared on ice using 0.2 mL nuclease free, thin walled PCR tubes. Each PCR tube contained the following: 2 x Reaction mix (dNTPs: 200µM; $MgSO_4$:1.6mM) 25µL, Template RNA (200 ng/µL) 1µl, Sense Primer (0.2µM), 1 µL, Antisense Primer (0.2µM)1µL, SuperScript III RT/ Platinum Taq mix (5 mM) 2µL, Autoclaved distilled water; 20 µL was added to make up total volume to 50 µL.

These were mixed together gently and all the components were allowed to settle at the bottom of the amplification tube. The tubes were then centrifuged briefly by pulse centrifugation, over-laid with one drop of mineral oil and placed in a preheated thermal cycler (GeneAMP PCR System) as programmed above. One tube was used as blank and contained only master mix and water.

Analysis of the RT-PCR products: The analysis of the PCR products was carried out using the following method. Agarose gel (2%) was made by dissolving 2.5 g agarose in 112.5 mL of distilled water which was subsequently micro-waved in a conical flask for 5 min. 12.5 mL buffer solution of Tris/Borate/EDTA (TBE) was added and 5 µL of ethidium bromide added for staining the mRNA and mixed thoroughly. The mixture was poured into the gel tray with combs in place and air bubbles pushed to the sides with a pipette. The gel was allowed 1 h to set. 5 µL of Blue loading buffer (Sigma, G7654), which contain bromphenol blue (0.25% w/v), xylene cyanole FF (0.25% w/v), sucrose (40% w/v), was added to each of the samples and centrifuged briefly to mix and settle.

A 10 µL DNA ladder (Promega, G2101) and 5 µL Blue loading buffer was used to make-up the DNA marker, and one litre of gel buffer was made using the TBE in a 1:10 dilution. The gel was placed in an electrophoresis tank with wells at the negative electrode such that RNA will move towards the positive electrode. The gel was then surrounded with gel buffer and the wells completely covered by the buffer. The DNA marker (5 µL), was added to the gel on position one, followed by adding a 10 µL blank sample to the next position, and continuing to add 10 µL of each sample to the remaining wells. The electrophoresis tank was connected to a power supply set at 125V for one hour. Photographs of the gels were taken and scanned using the digital densitometer to evaluate and semi-quantify the mRNA of the receptors, and then compared between the different groups.

Statistical analysis: The different parameters measured from the normal, hypoxic and treated experimental groups of B50 neuronal cells were compared using mean and standard deviation (SD). The parameters were assayed in triplicate and repeated twice (n = 6) and the results presented as the mean ±SD. The Students't-test was used for testing the level of significance between two groups and a p-value less than 0.05 was considered to be significant using Microsoft Excel® package. For multiple treatment data, One-Way Analysis of Variance (ANOVA) was used followed by Multiple Range Test post hoc subgroup testing to find the Least Significant Difference (LSD) between the groups.

RESULTS

Morphological studies: Morphological changes were observed in B50 cells cultured under hypoxia when compared to cells cultured in normoxia. The B50 cells in hypoxia showed clustered groups of neuronal B50 cells, evidence of degenerating, dying cells and already degenerated and dead neuronal B50 cells. The normal B50 neuronal cells cultured under normal incubator showed normal neuronal morphology (Plate 1 and 2), when compared to B50 cells in hypoxia (Plate 3 and 4).

The effect of hypoxia on the expression of Mu Opioid receptor gene in B50 cells: The result of Mu receptor gene expression in B50 neuronal cells in normal, hypoxic and treated cells was studied using reverse transcription

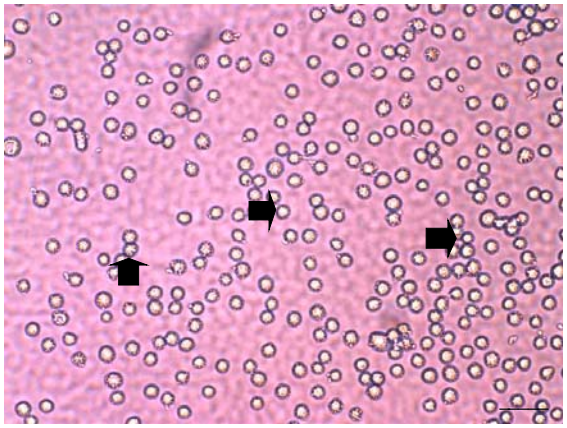

Plate 1: Representative of B50 cells at ohrs with normal B50 cells (arrow) at the point of starting the culture at 21% O2 and 5% CO2. B50 cells were observed in three different plates with same field method in a quadri-point analysis under the Nikon Eclipse Eclipse TS 100 microscope and microphotographs processed with an IBM Image Solution ®. Scale bar=5mm×40 magnification.

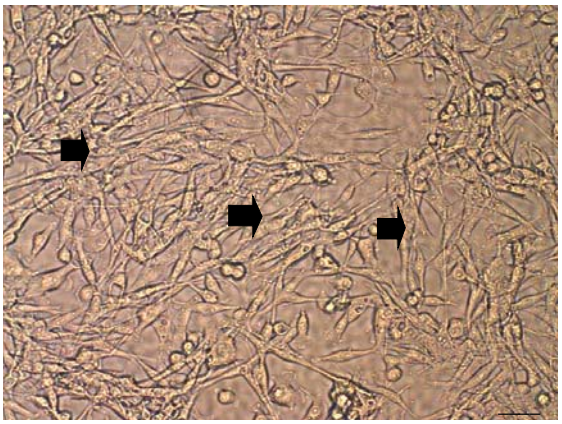

Plate 2: Representative of B50 cells at 48hrs of normal cultural (21%O2 and 5%Co2) with B50 cells (arrow). B50 cells were observed in three different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS 100 microscope and microphotographs processed with IBM Image Solution®. Scale bar=5mm×40 magnification.

polymerase chain reaction (RT-PCR). (Fig. 1). The results showed that the RT-PCR experiments with the B50 neuronal cells in normal, hypoxic and treated cultures demonstrated positive gene expression of the Mu opioid receptors. The mRNA levels of Mu opioid receptors in hypoxic culture of B50 cells were expressed relative to Mu opioid receptors in B50 cells cultured under normal conditions, and these were also compared with mRNA levels of Mu opioid receptors in hypoxic B50 cells treated with different receptor agonists (Fig. 2). The results showed that there were no significant difference in the

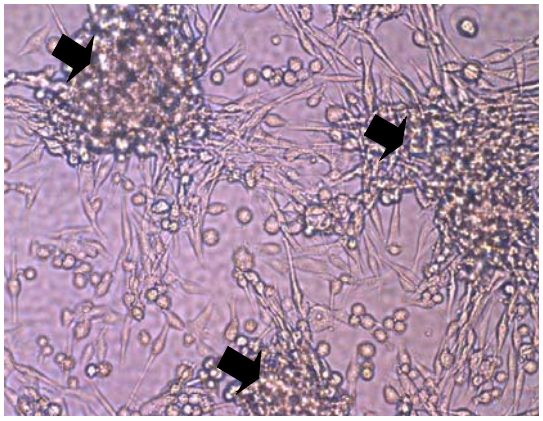

Plate 3: Representative of hypoxic B50 cells at 48hrs of culture (5% O_2 and 5%CO_2) with out drug administration, showing groups of degenerating cells (arrow). Groups of degeneration cells were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse Eclipse TS 100 microscope and microphotographs processed with an IBM Image Solution ®. Scale bar=5mm×40 magnification.

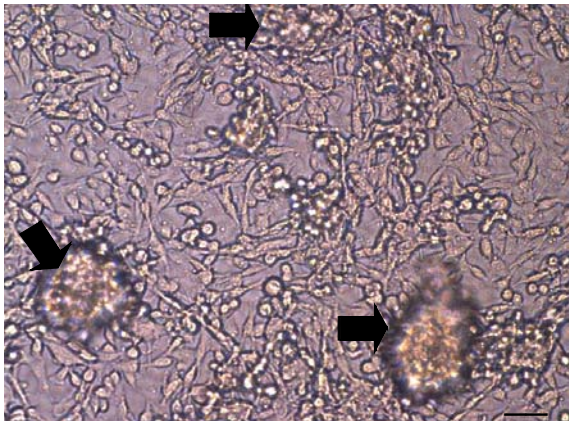

Plate 4: Representative of B50 cells in hypoxia at 96hrs at 96hrs of cultural (5% O2 and 5% Co2) with groups of degenerating cells (arrow). B50 cells was observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS 100 microscope and microphotographs processed with an IBM Image Solution ®. Scale bar=5mm×40 magnification.

levels of mu opioid receptor gene and mRNA expression between the normal, hypoxic and agonist treated cultured B50 cells.

Semi quantitative RT-PCR of cannabinoid CB_1 and MOR: MOR expression products were subjected to semi quantitative analysis using digital densitometric measurements. The decrease in the density of MOR

Table 1: Semi-Quantitative RT-PCR product of MOR receptors in B50 cells in culture

TreatmentType	Vol.mm^3	Areamm^2	STD ±	Density	Width	Height	Normal (%)	Control (%)
MOR Normal	11.20	4.04	0.32	621.12	2.78	1.56	100	119.57
MORHypoxia	04.55	1.94	0.24	583.40	0.76	2.54	93.93	112.31
10µM DAMGO	14.57	5.90	0.35	613.07	2.73	2.16	98.70	118.02
50µMDAMGO	12.48	5.34	0.34	579.21	1.59	3.37	93.27	111.50
100µMDAMGO	10.61	4.29	0.35	613.22	1.78	2.41	98.73	118.05
DAMGO/CTAP	11.29	4.44	0.33	631.09	2.79	1.59	101.61	121.49
Alpha actin	10.35	3.22	0.32	519.46	2.02	3.12	86.63	100

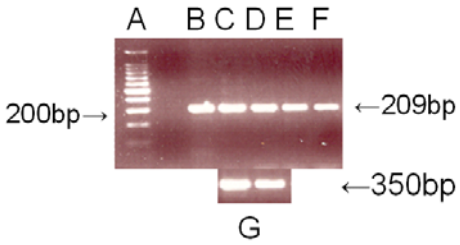

Fig. 1: The effect og opioid treatment on MOR expression in cultured B50 cells in hypoxia, A = DNA Ladder; B = Normal cells; C = Hypoxic cells; DEF = Hypoxic MOR treated; G = Alpha action

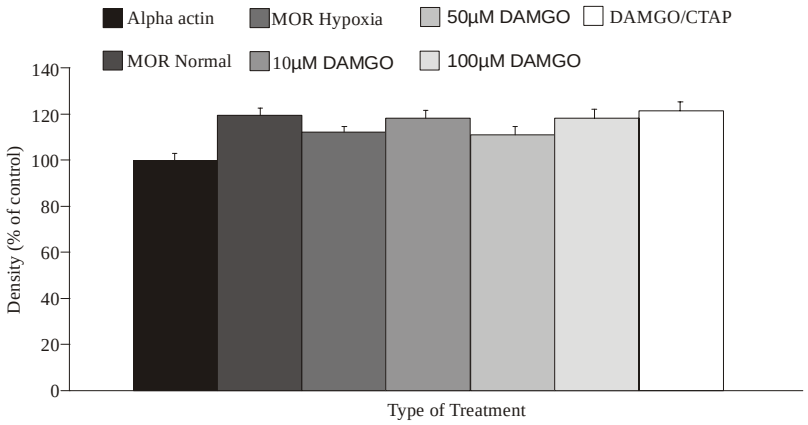

Fig. 2: The effect of hypoxia on the expression density of MOR receptor in B50 cells

RT-PCR products was not significant in untreated (MOR) hypoxic cells (93%) and those treated with 10 µM DAMGO (98%); 50 µM DAMGO and 100 µM DAMGO (98%). There was no significant change in densitometric measurements of mRNA area, mRNA volume, height and width respectively when compared with the normal control B50 cells (Table 1).

When the density of the RT-PCR products was normalised to alpha actin (100%), there was no observed difference in the level of the receptor mRNA expression of MOR in normal, hypoxic and treated cultured B50 cells. The results showed no significant difference between the normal MOR (119%); 10 µM DAMGO (118%); 50 µM DAMGO (111%) and 100 µM DAMGO (118%).

Mu opioid (MOR) receptor expression in B50 neuronal cells in culture. Total RNA was extracted from cultured B50 cells in normal (21% O_2, 5% CO_2), hypoxic (5% O_2, 5% CO_2) and hypoxia treated with MOR agonists. RT-PCR amplification was performed with specific MOR primers. The gel was electrophoresed and stained with ethidium bromide to visualise the intensity of the cDNA for MOR (209 bp) and was normalised relative to alpha actin (350 bp)

MOR receptor was expressed in B50 neuronal cells in culture with total RNA extracted from B50 cells in normal (21% O_2, 5% CO_2), hypoxic (5% O_2, 5% CO_2) and hypoxia treated with MOR agonists. The RT-PCR was amplified with specific MOR primers and gel was electrophoresed and stained with ethidium bromide and normalised relative to alpha actin. The RT-PCR product was quantified with digital densitometer and expressed as percent of control, (Data presented as mean ±SD; Student's t-test).

MOR receptor was expressed in B50 neuronal cells in culture with total RNA extracted from B50 cells in normal (21% O_2, 5% CO_2), hypoxic (5% O_2, 5% CO_2) and hypoxia treated with MOR agonists. The RT-PCR was amplified with specific MOR primers and gel was electrophoresed and stained with ethidium bromide and normalised relative to alpha actin. The RT-PCR product was quantified with digital densitometer and expressed as percent of control, (Data presented as mean ±SD; Student's t-test)

DISCUSSION

The use of experimental approaches which include morphological assessments, cell live and dead trypan staining and viability and proliferation assay was used to assess neuronal injury more directly from hypoxia. It has been shown that during hypoxia, glutamate is expelled from neurons leading to over-stimulation of glutamate receptors and subsequent injury and death of the neurons (Haddad and Jiang, 1993; Nyakas *et al*., 1996). Since Zhang *et al*. (2002) had shown that because glutamate receptor expression increases during development, and sensitivity to glutamate excitotoxicity increases with neuronal maturation, the observed differences in hypoxic susceptibility between the neuronal ages in this study may be associated with the developmental increase in glutamate toxicity (Choi and Rothman, 1990).

This study has demonstrated that stimulation of opioid receptors, proffers some protection by reducing neuronal cell injuries and deaths after treatments in hypoxic conditions but the benefit is reduced substantially with prolonged exposure durations and higher concentrations of the drugs. This supports the finding that the longer the neurons stayed in the culture media both in the normal and in the experimental hypoxic groups, the greater the extent of the neuronal cell injuries and death. A possible explanation for this phenomenon is that prolonged hypoxia may cause a significant release and accumulation of endogenous glutamate which causes glutamate-induced toxicity and hence death of the cells (Nyakas *et al*., 1996). Another alternative explanation is that prolonged hypoxia causes the release and accumulation of endogenous opioids which saturates opioid receptors in these neurons. Hence the positive effect of the opioid agonists decreases with the increase in the time the cells stay in the medium which may result in desensitization of the receptors and the reduced effect of the action of the drugs (Mao *et al*., 2002; Wallace *et al*., 2006).

It has been shown that in response to short-term hypoxia, the level of enkephalins, the endogenous agonists for opioid receptors sharply increases (Zhang *et al*., 2002; Khasabova *et al*., 2002). This showed that cortical neurons may release opioids during normal function and in response to hypoxic stress as a mechanism of self-protection against injury (Ma *et al*., 2005; Wallace *et al*., 2006). Zhang *et al*. (2002) showed that because of high levels of endogenous opioids which may already be present in the culture media after prolonged exposure to hypoxia, adding more agonist may not increase the protection by the drugs. On the other hand, desensitization of the receptors may have occurred due to prolonged treatment of the cells with the agonists in conjunction with the endogenous opioid release during chronic hypoxia. Zhang *et al*. (2006) have shown that cortical neurons are highly susceptible to opioid receptor inhibition, which causes serious neuronal injury especially during hypoxic stress.

However, cellular injury was observed in cultured B50 neurons than in both normoxic and hypoxic conditions which suggests that maturational differences exist between this age group of neurons. This supports the work of Zhang *et al*. (2000), which showed that opioid receptor expression increases significantly with development in both brain and cultured neurons. This is because the opioid receptor density increases with increase in the age of the neurons though the result from this present study shows that there was no difference in the mu opioid receptor gene expression and no significant change in the expression densities of the mu opioid receptors. The more mature neurons may have greater dependence on this pathway to maintain neuronal function and therefore may be more susceptible to neuronal injury with opioid receptor inhibition. This observation suggests that the increase in opioid receptor agonist may compete with opioid receptor antagonist in terms of opioid receptor binding and thus reduce neuronal injury induced by opioid receptor inhibition during prolonged hypoxia (Wallace *et al*., 2006). This is in support of the findings in this study in which at higher concentration of the agonists and antagonist during the treatment against hypoxia, led to complete inhibition of the agonists activities in cultured B50 cells in hypoxia.

Another issue is whether the differences in expression levels of the various opioid receptors account for the observed phenomenon in cortical neurons. Past studies have demonstrated that μ-opioid receptors are present at similar or even higher densities than δ-opioid receptors in mammalian cortex, although κ-opioid receptor density is slightly lower (Zhang *et al*., 2006). This implies that the relative distribution and expression levels of opioid receptor subtypes within the cortex as a whole may not be a key factor in the observations shown in this work. This is because the results presented in this study show that there was no observed differences in the receptor expression densities of μ-opioid receptors in normoxic, hypoxic and hypoxic treated B50 cells in culture.

The mechanisms of opioid receptor neuroprotection activity may involve the regulation of specific G proteins, ion channels mainly Ca^{2+} and K^{+} channels and excitatory

neurotransmitter release. It has been shown that intracellular Ca^{2+} levels are elevated during hypoxic exposure leading to irreversible cell injury while the inhibition of Ca^{2+} currents by opioid receptor stimulation by DAMGO, DSLET and ICI-199441, may serve as a neuroprotective mechanism in preventing Ca^{2+} overload (Andersen, 2004; Bossy-Wetzel *et al*., 2004). Also opioid receptor regulation of glutamate signalling may be involved in normal function and protection of neurons (Ma *et al*., 2005, Mao *et al*., 2002). It has also been shown that opioid receptor agonists have the ability to reduce neuronal over-stimulation by blocking glutamate excitation (Zhang *et al*., 2002), and this could be the situation with the protection proffered by the μ-, δ- and κ-opioid agonists used in this study. This mechanism of cellular regulation may be utilised during normal cell functioning and in response to environmental stress like hypoxia (Andersen, 2004). Zhang *et al*. (2006) have shown that the inhibition of opioid receptors in normal neurons may lead to substantial injury in the neurons by the loss of inhibitory regulation of the excitatory neurotransmitter release and/or receptor excitation. The δ-, μ- and κ-opioid receptors have many similarities such as seven transmembrane domains existing as 60% identical sequences and being coupled to $G_{i/o}$ proteins. Connor and Christie (1999) had proposed that because of the common features of opioid receptors, the selectivity of these receptors for eliciting specific pathways does not lie in the differences between each opioid receptor subtype but in their association with other divergent types of G proteins. It was observed that each opioid receptor subtype also preferentially couples to specific G proteins apart from the $G_{i/o}$ proteins that they generally couple (Zhang *et al*., 2002). Examples of this preferential coupling include as seen in δ-opioid receptors which are more efficiently coupled to $G\alpha_{16}$ protein than either μ- or κ-opioid receptors (Lee *et al*., 1998). Also μ-receptor agonist (DAMGO) has been reported to have a selective coupling to $G\alpha_{i1}$ and $G\alpha_{oA}$ opioid receptors than other opioid agonists (Saidak *et al*., 2006). This shows that the opioid receptor agonists have selective activation of G-proteins in response to opioid receptor activation. This preferential coupling to other G protein subtypes may be the reason for the observed differences in the effect of the opioid agonists on the B50 cells treated in hypoxia.

CONCLUSION

The results from the present study showed that hypoxia induced morphological changes in B50 cells in hypoxia while the Mu RT-PCR mRNA levels showed no appreciable changes in normal, hypoxic and cells treated with Mu opioid receptor agonists. The results show that B50 neuronal cells are susceptible to damage and injurious effects of hypoxia, as are most brain cells and the Mu opioid receptor agonist treatments showed that there were no changes in the level of Mu opioid receptor gene expression due to hypoxia or agonist treatment in neuronal B50 cells in culture.

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REFERENCES

Andersen, B.B., 2004. Reduction of Purkinje cell volume in cerebellum of alcoholics. Brain Res., 1007(1-2): 10-18.

Bossy-Wetzel, E., M.V. Talantova, W.D. Lee, M.N. Scholzke, A. Harrop, E. Mathews, T. Gotz, J. Han, M.H. Ellisman, G.A. Perkins and S.A. Lipton, 2004. Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p38-activated K^+ channels. Neuron, 41(3): 351-365.

Boutilier, R.G., 2001. Mechanisms of cell survival in hypoxia and hypothermia. J. Exp. Biol., 204(Pt 18): 3171-3181.

Chaturvedi, K., K.H. Christoffers, K. Singh and R.D. Howells, 2000. Structure and regulation of opioid receptors. Biopolymers, 55(4): 334-346.

Choi, D.W. and S.M. Rothman, 1990. The Role of Glutamate Neurotoxicity in hypoxic-ischaemic neuronal death. Annu. Rev. Neurosci., 13: 171-182.

Connor, M. and M.D. Christie, 1999. Opioid receptor signalling mechanisms. Clin. Exp. Pharmacol. Physiol., 26(7): 493-499.

Corbett, A.D., G. Henderson, A.T. McKnight and S.J. Paterson, 2006. 75 years of opioid research: the exciting but vain quest for the Holy Grail. Br. J. Pharmacol., 147: S153-S162.

Dietz, A., B. Vanselow, V. Rudat, C. Conradt, H. Weidauer, Kallinowski and R. Dollner, 2003. Prognostic impact of reoxygenation in advanced cancer of the head and neck during the initial course of chemo radiation or radiotherapy alone. Head Neck, 25(1): 50.

Fichna, J., K. Gach, M. Piestrzeniewicz, E. Burgeon, J. Poels, J.V. Broeck and A. Janecka, 2006. Functional characterization of opioid receptor ligands by aequorin luminescence-based calcium assay. J. Pharmacol. Exp. Ther., *317*(3): 1150-1154.

Guyton, A.C. and J.E. Hall, 2005. Textbook of Medical Physiology with Student consult Access. 11th Edn., W.B. Saunders Co. Philadelphia.

Haddad, G.G. and C. Jiang, 1993. O_2 deprivation in the central nervous system: on mechanisms of neuronal response, differential sensitivity and injury. Prog Neurobiol., 40(3): 277-318.

Hasbi, A., S. Allouche, F. Sichel, L. Stanasila, D. Massotte, G. Landemore, J. Polastron and P. Jauzac, 2000. Internalization and recycling of delta-opioid receptor are dependent on a phosphorylation-dephosphorylation mechanism. J. Pharmacol. Exp. Ther., 293(1): 237-247.

Hochachka, P.W., 1986. Defense strategies against hypoxia and hypothermia. Science, 231(4735): 234-241.

Khasabova, I.A., D.A. Simone and V.S. Seybold, 2002. Cannabinoids attenuate depolarization -dependent Ca^{2+} influx in intermediate-size primary afferent neurons of adult rats. Neuroscience, 115(2): 613-625.

Laugwitz, K.L., S. Offermanns, K. Spicher and G. Schultz, 1993. μ and δ opioid receptors differentially couple to G protein subtypes in membranes of human neuroblastoma SH-SY5Y cells. Neuron, 10(2): 233-242.

Ma, M.C., H. Qian, F. Ghassemi, P. Zhao and Y. Xia, 2005. Oxygen-sensitive δ-opioid receptor-regulated survival and death signals: novel insights into neuronal preconditioning and protection. J. Biol. Chem., 280: 16208-16218.

Mao, J., B. Sung, R.R. Ji and G. Lim, 2002. Neuronal apoptosis associated with morphine tolerance: evidence for an opioid-induced neurotoxic mechanism. J. Neurosci., 22(17): 7650-7661.

Lee, J.W., S. Joshi, J.S. Chan and Y.H. Wong, 1998. Differential coupling of mu-, delta-, and kappa-opioid receptors to G alpha16-mediated stimulation of phospholipase C. J. Neurochem., 70(5): 2203-2211.

Mahura, I.S., 2003. Cerebral ischemia-hypoxia and biophysical mechanisms of neurodegeneration and neuroprotection effects. Fiziol Zh., 49(2): 7-12.

Massotte, D. and B.L. Kieffer, 1998. A molecular basis for opiate action. Essays Biochem., 33: 65-77.

Milligan, G., 2004. G protein-coupled receptor dimerization: Function and ligand pharmacology. Mol. Pharmacol., 66: 1-7.

Nyakas, C., B. Buwalda and P.G. Luiten, 1996. Hypoxia and brain development. Prog Neurobiol., 49(1): 1-51.

Park, F., D.L. Mattson, L.A. Roberts and A.W. Cowley, 1997. Evidence for the presence of smooth muscle alpha-actin within pericytes of the renal medulla. Am. J. Physiol., 273(5 Pt 2): R1742-R1748.

Piestrzeniewicz, M.K., J. Michna and A. Janecka, 2006. Opioid receptors and their selective ligands. Postepy Biochem., 52(3): 313-319.

Priebe, L., M. Friedrich and K. Benndorf, 1996. Functional interaction between K_{ATP} channels and the Na^+–K^+ pump in metabolically inhibited heart cells of the guinea pig. J. Physiol., Lond., 492: 405-417.

Raynor, K., H. Kong, S. Law, J. Heerding, M. Tallent, F. Livingston, J. Hines and T. Reisine, 1996. Molecular biology of opioid receptors. NIDA Res. Monogr., 61: 83-103.

Reisine, T. and G.I. Bell, 1993. Molecular biology of opioid receptors. Trends Neurosci., 16(12): 506-510.

Reisine, T. and M.J. Brownstein, 1994. Opioid and cannabinoid receptors. Curr. Opin. Neurobiol., 4(3): 406-412.

Rhim, H. and R.J. Miller, 1994. Opioid receptors modulate diverse types of calcium channels in the nucleus tractus solitarius of the rat. J. Neurosci., 14(12): 7608-7615.

Rolfe, D.F.S. and M.D. Brand, 1996. Contribution of mitochondrial proton leak to skeletal muscle respiration and to standard metabolic rate. Am. J. Physiol., 271: C1380-C1389.

Saidak, Z., K. Blake-Palmer , D.L. Hay, J.K. Northup and M. Glass, 2006. Differential activation of G-proteins by μ-opioid receptor agonists. Br. J. Pharmacol., 147(6): 671-680.

Silbert, S.C., D.W. Beacham and E.W. McCleskey, 2003. Quantitative single-cell differences in mu-opioid receptor mRNA distinguish myelinated and unmyelinated nociceptors. J. Neurosci., 23(1): 34-42.

Tomaselli, B., V. Podhraski, V. Heftberger, G. Bock and G. Baier-Bitterlich, 2005. Purine nucleoside-mediated protection of chemical hypoxia-induced neuronal injuries involves p42/44 MAPK activation. Neurochem. Int., 46(7): 513-521.

Tso, P.H. and Y.H. Wong, 2000. G(z) can mediate the acute actions of mu- and kappa -opioids but is not involved in opioid-induced adenylyl cyclase super sensitization. J. Pharmacol. Exp. Ther., 295(1): 168-176.

Wallace, D.R., S.L. Dodson, A. Nath and R.M. Booze, 2006. Delta opioid agonists attenuate TAT_{1-72}-induced oxidative stress in SK-N-SH cells. Neuro Toxicol., 27(1): 101-107.

Wang, J., Q. Gao, J. Shen, T.M. Ye and Q. Xia, 2007. Kappa-opioid receptor mediates the cardio protective effect of ischemic post conditioning. Zhejiang Da Xue Xue Bao Yi Xue Ban, 36(1): 41-47.

Zhang, J., G.T. Gibney, P. Zhao and Y. Xia, 2002. Neuroprotective role of delta-opioid receptors in cortical neurons. Am. J. Physiol. Cell Physiol., 282: C1225-C1234.

Zhang, J., G.G. Haddad and Y. Xia, 2000. δ-, but not μ- and κ-, opioid receptor activation protects neocortical neurons from glutamate-induced excitotoxic injury. Brain Res., 885: 143-153.

Zhang, J., H. Qian, P. Zhao, S.S. Hong and Y. Xia, 2006. Rapid hypoxia preconditioning protects cortical neurons from glutamate toxicity through delta-opioid receptor. Stroke, 37(4): 1094-1099.